STAIN TECHNOLOGY

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ANNOUNCEMENTS

MEETING OF THE EXECUTIVE COMMITTEE OF THE BIOLOGICAL STAIN COMMISSION

During the early years of the Commission frequent meetings of the Executive Committee were held. For over ten years, however, this practice has been discontinued, partly because of financial difficulties and partly because after completing the organization of the Commission's work, there was not enough business to justify getting the Commission together.

This year, at last, another meeting of the Committee has been held and it has been decided to hold such meetings more frequently in the future. The meeting this year was held in Philadelphia, February 12th and all but one of the members were present. At this meeting Dr. Gershenfeld was elected Secretary of the Commission, thus making the officers of this organization the following:

Chairman:	H. J. Conn
Vice chairman and Treasurer:	S. I. Kornhauser
Secretary:	Louis Gershenfeld

The chairman presented a financial report showing that war conditions have not only enabled the Commission finances to show a surplus instead of a deficit, but are permitting the accumulation of a reserve fund with which to offset deficits that may occur during the years following the war. The accumulation of this reserve fund, however, is forcing on the Commission certain matters of reorganization to assure keeping the funds in such a way as to be safe and yet readily available when needed. As a first step the Executive Committee appointed a smaller finance committee with powers to act in all financial matters; for the present the finance committee consists of the three above-named officers of the Commission.

It was also resolved that the officers should constitute a committee to consider whether the Constitution of the Commission should be revised and if so to draw up suggestions for its revision. The present Constitution[1] was drawn up twenty years ago; and altho occasionally amended[2], it is hardly up to date, and may well need complete revision. When it was written the Commission was an almost un-

[1]Stain Techn., **1**, 4–8, 1926.

[2]Stain Techn., **4**, 71, 1929; **7**, 75, 1932.

known organization wholly dependent on outside sources of money to keep it going; now it has an independent income and is sufficiently well known so that the Army and Navy both require its certification on stains which they purchase. Such a complete change in the character of the organization was by no means anticipated at the outset; and a Constitution drawn up then may well be out of date under present conditions.

Considerable attention was given to the question whether or not the Stain Commission should incorporate. When first organized the Commission officers were advised not to incorporate. Present circumstances have completely changed the situation. Considerable property is now held in the name of the Commission which cannot be legally owned by it without incorporating. Accordingly the Committee instructed the Chairman to look into the matter seriously and to report to the other officers what changes in organization would be necessary in order to take this step.

As plans for the reorganization of the Commission and the revision of its Constitution develop, announcements will be made in these pages.

Besides such matters of business, the Committee discussed numerous subjects of more general nature. The present great demand for certified stains was mentioned, and the question was raised as to where they are going. It is known that the bulk of present purchases are by the Government; but it is not known whether they are being ordered for our armed forces alone or whether they may not in part be distributed to other countries thru the Government's lend-lease agencies. The latter possibility was regarded as especially interesting because of the chances it would offer for advertising Commission certified stains thruout the world. The Chairman and Secretary were instructed to make inquiries on the subject.

The possibility of making the work of the Stain Commission better known to Latin American users of stains was also discussed. This matter was referred to Dr. R. D. Lillie because of his connection with the Pan American Sanitary Bureau.

The meeting adjourned after a motion had been made, seconded and carried that annual meetings be held in the future.

A New Commission Publication

Announcement is now made of a new Commission-sponsored publication to be known as Staining Procedures (compiled by H. J. Conn and Mary A. Darrow, with the assistance of numerous collaborators). It is to be a loose-leaf handbook, consisting eventually of perhaps ten leaflets, each of which is to be revised from time to time to keep it up to date.

At the present time only the first two leaflets are ready for publication: an introductory section dealing with general methods, fixing fluids, etc.; and one on methods for staining general tissue in animal histology. A preliminary draft of each section has been submitted to Commission members, and thanks are hereby extended to all those who have submitted suggestions for improvement. Because of this coöperation, the methods thus published can be regarded, not as selected by one or two individuals, but as endorsed by a considerable body of biologists.

A publication notice of these first two leaflets is given in the advertising section of this issue.

COLCHICINE-FEULGEN LEAF SMEARS

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ABSTRACT.—**The colchicine-Feulgen leaf smear has many advantages. It (1) prevents spindle formation, (2) allows the chromosomes to be widely spread in the cell, (3) straightens the chromatids, (4) allows the constrictions to become very noticeable, (5) increases the number of chromosome plates by preventing anaphase, (6) facilitates smearing, and (7) stains only the chromosomes. (8) Young leaves are generally easily obtainable, while roots of the proper sort are to be had only under very special conditions. (9) Mitoses are frequently more numerous in young leaves than in roots. The schedule for specimens of *Phlox* involves a 1 to 2 hour pretreatment of young leaves in a 0.2% colchicine solution, fixation in Semmen's Carnoy (3 vol. absolute alcohol, 1 vol. glacial acetic, 1 vol. chloroform), hydrolysis for 25 minutes in 10% HCl at 58° C., staining in decolorized fuchsin, smearing in 45% acetic acid, and running the material on slide and cover glass thru acetic alcohols (1:1, 1:3, 1:9), absolute alcohol, xylol, and balsam.**

Colchicine pretreatment has been used by several investigators in the smearing of root tips for chromosome studies. O'Mara (1939) showed that as a method of studying the morphology of somatic chromosomes, it yielded results far superior to either sections or ordinary smears. Burrell (1939) suggested the use of colchicine to shorten the chromosomes of certain plants, thus making the chromosomes easier to count. Both of these workers used aceto-carmine. Bhaduri (1940) and Nichols (1941) used colchicine treatment and the Feulgen reaction in the making of root-tip smears.

Baldwin (1939) used aceto-carmine smears of young leaves and first brought them to this writer's attention as a possible source of mitotic material in specimens of *Phlox*. A very successful technic has been developed which involves colchicine pretreatment, Feulgen staining, and the smearing of very young leaves. This method has been used only with species of *Phlox*, but it has given such consistently good results that it should prove useful in the study of chromosome number and morphology of other plants.

There are many advantages of the method. The collection of leaves is quicker, simpler, and less disturbing to the plant than is the digging of roots. Since it is less harmful to the plant, the permission to collect young leaves from choice plants is more easily obtainable. In addition, the leaves often seem to show greater mitotic activity than do the roots. The colchicine pretreatment prevents spindle formation and allows the chromosomes to be widely spread thruout the cell. The chromatids in pretreated leaves shorten and thicken, lose their relational coiling and become straight; their constrictions become very apparent. (See Figure 1.) This spreading, loss of relational coiling, and straightening allows the chromosomes to be placed in almost a single plane by pressure on the cover glass during smearing. One of the greatest advantages is that colchicine prevents anaphase, and this results in many more metaphase-like figures in treated than in untreated material. The Feulgen technic facilitates smearing and stains only the chromosomes.

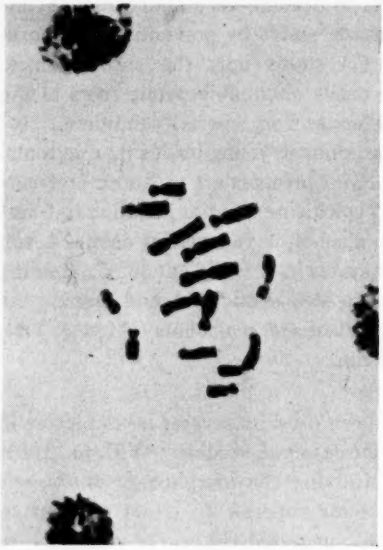

FIG. 1. Photograph of a mitotic plate of *Phlox paniculata* Katherine (1670×) from a colchicine Feulgen leaf smear.

In the laboratory ½ inch or less of the desired percentage of aqueous colchicine solution may be put into shell vials, and the vials put into a small, low box for carrying to the field. There the vials may be taken out one at a time and labelled with a wax pencil as the material is collected. The box of vials is then brought into the laboratory and placed under a gooseneck lamp with a 60 or 100 watt bulb; this may, or may not, be important. Care must be taken not to let the material transpire so fast that it wilts.

Only healthy, young, fast-growing leaves should be used. In *Phlox subulata* or *P. nivalis*, leaves about 1 mm. long have abundant divisions, while in *P. paniculata* 4 mm. leaves are suitable. Stem

tips bearing these leaves should be quickly cut off with a sharp razor blade and immediately dropped and immersed, cut end down, in the solution in a vial.

The optimum percentage of colchicine and time of treatment may vary with the material.

The schedule for *Phlox* is as follows:

1. Quickly remove young, fast-growing stem tips with a sharp razor blade and immerse them in 0.2% colchicine solution.
2. Pretreat the material, in light, for 1–2 hours.
3. Remove older, outer leaves to expose the youngest leaves. Blot the colchicine solution from the material.
4. Fix material (young leaves and stem) in Semmen's Carnoy (3 parts absolute alcohol : 1 part glacial acetic acid : 1 part chloroform).
5. After 6–24 hours, blot off most of the fixative and put material into cold 10% HCl (1 part conc. HCl : 9 parts water).
6. After material sinks, transfer it into a corked vial of warmed 10% HCl in a 58° C. oven.
7. Hydrolize for 25 minutes.
8. Rinse twice in distilled water.
9. Transfer to decolorized fuchsin solution. Leave 20–30 minutes.
10. Put material on a *clean* slide. Rinse with 45% acetic acid. The acid is applied from a dropper bottle and removed with pieces of absorbent paper toweling.
11. Dissect out the darkest stained tissue, discarding the rest.
12. Rinse selected tissue several times with 45% acetic acid.
13. Macerate the tissue in a drop of 45% acetic acid until it is a pulp. This may be accomplished by tapping it with the flat side of a spear needle.
14. Add a *clean* cover glass, and apply pressure in a slide-blotting book. The slide may be examined now, by using 45% acetic acid at the cover glass edge to prevent drying. The best mitotic plates are usually near the edge of the cover glass.
15. Invert slide in acetic alcohol (1:1) in a petri dish. Use glass supports under the ends of the slide. If the cover glass does not fall to the bottom of the petri dish in 30 minutes, carefully work if off the slide with needles.
16. Transfer slide and cover glass to acetic alcohol (1:3) for 5 minutes.
17. Transfer to acetic alcohol (1:9) for 5 minutes.
18. Transfer to absolute alcohol for 5 minutes. (Good results have been obtained by omitting steps 16 and 17.)
19. Transfer to xylol for 5 minutes.
20. Mount in balsam, recombining slide and cover glass.

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A SPECTROPHOTOMETRIC ANALYSIS OF TISSUE STAINING[1]

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ABSTRACT.—**By comparing spectral absorption curves of representative staining solutions and of substances stained with these solutions it is shown that information may be obtained regarding chemical changes associated with the staining process. The stains used in these determinations were acid fuchsin, anilin blue, azocarmine G, basic fuchsin, eosin Y, orange G, picric acid and Sudan IV. The substrates stained were gelatin, tendon, blood plasma, thymus gland and fat.**

Aqueous basic fuchsin and fuchsin-sulfurous reagent to which formalin was added (Schiff reaction) are different stains. The spectral absorption curves for staining solutions and substances stained with the solutions were comparable. Within the limitations of the spectrophotometric methods and stains employed, there was no evidence of significant chemical alteration in the chromophore radicals of the stains associated with the process of tissue staining.

The changes in epidermal cells which become malignant following treatment with methylcholanthrene are being investigated at the Barnard Hospital under the direction of Dr. E. V. Cowdry. Those properties which can be measured quantitatively are obviously the best suited for such an integrative study. For the quantitative photometric study of stained tissue elements (Stowell, 1942), it is important to determine the absorption spectra of specifically stained tissues.

A comparison of the absorption spectra of staining solutions with that of the various tissues with which they have been stained, may furnish information regarding the chemical or physical nature of the process of tissue staining. If the absorption spectrum of a staining solution and of a stained tissue are the same, it is reliable evidence that

[1]This investigation was aided by a grant from an anonymous donor.

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the chromophore group of the dye has not undergone appreciable chemical change. In this paper the absorption spectra of certain commonly used staining solutions are compared with the absorption of stained gelatin, tendon, blood plasma, thymus and fat.

Preparation of Stains and Tissues

The stains, tissues stained and staining procedures employed are outlined in Table 1. Since it is not feasible to make careful determinations upon large numbers of dyes, the stains used in this study

Table 1. Stains, Procedures and Tissues Stained

Stain	Certification No. and dye content*	Tissue stained	Staining procedure
1. Acid fuchsin	NR 11; 62%	Gelatin Tendon Blood plasma Thymus	Mallory, p. 93 Mallory, p. 93 Mallory, p. 153 Mallory, p. 153
2. Anilin blue (water soluble)	NK 4; ——	Gelatin Tendon	Mallory, p. 154 Mallory, p. 154
3. Azocarmine G	———; 83%	Blood plasma Thymus	Mallory, p. 154 Mallory, p. 154
4. Basic fuchsin	NF 33; 87%	Thymus	Feulgen reaction, Stowell (1942)
5. Eosin Y	NE 12; 91%	Gelatin Tendon Blood plasma	0.5% alcoholic solution
6. Hematoxylin	FH 18; ——	Thymus	See text
7. Orange G	NO 7; 87%	Blood plasma	Mallory, p. 154
8. Picric acid	J. T. Baker Co.; C.P.	Blood plasma	Mallory, p. 93
9. Sudan IV	NZ 11; 86%	Human fat	Mallory, p. 118

*All dyes, except picric acid, were recent products of the National Aniline & Chemical Co., Inc.

were selected as representatives of several chemical groups, widely used in various combinations in histological staining. The nitro, quinone-imine and xanthene types of dyes are represented in this table by picric acid, azocarmine G and eosin Y respectively. Orange G and Sudan IV are azo dyes. Anilin blue and acid and basic fuchsin belong to the triamino tri-phenyl methane types of dyes. Hematoxylin is a natural dye. All of the stains except basic fuchsin and hematoxylin are acid dyes. These stains are employed in the most commonly used technics such as Harris' hematoxylin and

eosin, Van Gieson connective tissue stain, Mallory's triple connective tissue stain, Heidenhain's azocarmine stain, the Feulgen technic and fat stains.

The various substrates for nuclear and cytoplasmic staining were selected because of their relative purity of composition and simplicity of structure. Gelatin is the chief extractive protein in tendon. In the purified form used, gelatin is a relatively simple substrate for connective tissue stains. Tendon itself contains an insignificantly small amount of nuclear material. Concentrated blood plasma represents a relatively simple substrate for testing cytoplasmic stains. The isolation of pure nuclear material presents certain technical difficulties, but fortunately thymus gland contains relatively small amounts of cytoplasmic and reticular substances.

The various fixatives were selected on the basis of their simplicity of composition, of satisfactory fixation of the particular substrate, and in the case of sublimate-alcohol because of the desire to obtain information useful in other studies (Stowell, 1942).

A 10% aqueous solution of the "de-ashed gelatin" of Eastman Kodak Co. was fixed for 24 hours in a 10% solution of Merck's "Neutral Reagent" formalin and stored overnight in 80% ethyl alcohol. The Achilles tendon of a rabbit was fixed 24 hours in 10% formalin. A saturated aqueous solution of dried ox plasma, sold by Coleman & Bell Co. as "blood albumin," was placed in a filter paper sac, fixed for 24 hours in Bouin's fluid, and stored for several days in 70% alcohol. Thymus gland of a rabbit was fixed in equal parts of a saturated aqueous solution of $HgCl_2$ and of 95% alcohol for 18 hours. It was then rinsed in 70% alcohol. All tissues were dehydrated in ethyl alcohol, cleared in chloroform, embedded in paraffin (see Stowell, 1941, for schedule), sectioned at thicknesses of 10 and 20 μ, and mounted with 60% clarite in xylene. The slides and cover glasses were of measured, uniform thickness and of standard quality. Subcutaneous human fat, obtained following radical mastectomy, was extracted with alcohol-ether, washed with 10% formalin, stained with Sudan IV and rinsed in 70% alcohol. The sample of stained fat was large enough to permit measurements using standard absorption cells.

The staining solutions were prepared according to standard formulae, the references for which are given in Table 1. Only one dye was placed in each staining solution. For instance, the anilin blue and orange G solutions were prepared by dissolving 0.5 g. and 2.0 g. respectively of the dyes in separate 100 cc. portions of water to which 8 cc. of glacial acetic acid had been added. In general the maximum

time suggested in the references was used in staining the slides. Since we were interested in the staining of only one tissue component, the steps of the procedures concerned with the application of dyes other than the one with which we were immediately concerned were deleted from the staining schedules. That is, in applying the anilin blue stain steps No. 1 to 4 on pages 154–155 of Mallory's "Pathological Technique" (1938), which are concerned with the azocarmine nuclear stain, were deleted and the staining solution contained anilin blue, but not orange G. Stains were prepared 24 hours before the sections were stained, and mounted slides were kept in the dark two weeks before being measured.

Basic fuchsin is used in the Schiff reaction as a test for aldehydes. Feulgen and Rossenbeck (1924) found that their adaptation of this reaction is a relatively specific stain for thymonucleic acid in the nuclei of cells. In their procedure, basic fuchsin is reduced and decolorized by the action of SO_2. When tissue, which has been partially hydrolyzed with acid, is immersed in this fuchsin-sulfurous reagent the portions of the nucleus containing thymonucleic acid stain reddish-purple.

Three absorption curves were determined for basic fuchsin. Measurements were made on a 0.5% aqueous solution of the dye containing 10 cc. of *N* HCl to each 100 cc. of the staining solution. The dye was then converted to the colorless form by the action of $K_2S_2O_5$. After adding 2 drops of 40% formaldehyde to 10 cc. of the solution, as in the Schiff's reaction, absorption measurements were made. A third set of measurements were made on thymus tissue stained by a modified Feulgen technic (Stowell, 1942).

The Harris' hematoxylin staining solution was prepared according to Johansen (1940, p. 52). The slides were stained in the regular acidic, alcoholic solution and then made blue by treatment in 50% alcohol which had been adjusted to a pH of 8 by the addition of Li_2CO_3. It was not feasible to compare the color of these slides with the staining solution adjusted to a pH of 8 by Li_2CO_3 because of the significant precipitation of dye from the alkaline solution. Therefore, after several attempts, the work on hematoxylin was abandoned.

Methods of Measuring Absorption Spectra

The spectra of all of the stained sections were measured on a Model 10S Coleman Double Monochromator Spectrophotometer equipped with slits which isolate a spectral region 5 mμ in width. The spectrophotometer is used in conjunction with a Coleman model 310 Electrometer for measuring transmittance values directly. This instru-

ment is designed for use with a pair of matched absorption cells for measuring absorption spectra of solutions. The absorption cells are mounted in a special cell holder in the instrument, one cell containing the solvent and the other the solution. The mount for the cell holder is such that either of the cells can be moved into the light path between the exit slit of the monochromator and a photoelectric cell. The spectrophotometer is equipped with a vacuum type photoelectric cell with a variable resistance in its circuit. The photoelectric cell current develops a potential difference across this resistance which is proportional to the light intensity at a given wavelength and proportional to the value of the resistance used in the circuit. The electrometer consists of a uniform potentiometer with its dial calibrated from zero to 130 arbitrary units. A battery supplies a fixed steady current thru the potentiometer which produces a potential drop at its output proportional to the reading of the potentiometer dial. The output of the photoelectric cell circuit is connected to the electrometer so that the potential developed across the resistance in the photoelectric cell circuit is opposite to that developed across the section of the potentiometer, indicated by its dial as being in the circuit. A thermionic amplifier with a galvanometer in its plate circuit is used to indicate when these two potentials are equal.

The usual procedure in measuring the transmittance of a solution is to (1) set the wave-length drum on the spectrophotometer at the desired wave-length reading, (2) move the cell holder so that the cell containing the solvent is in the light path, (3) set the potentiometer at a reading of 100 units on its scale, (4) adjust the resistance in the photoelectric cell circuit until the galvanometer indicates a balance, (5) shift the cell holder so that the cell containing the solution is in the light path, and (6) adjust the potentiometer until the galvanometer again indicates a balance. The new reading of the potentiometer is equal to the transmittance of the solution.

In order to measure the transmittance of a stained section relative to a similarly prepared unstained section, it was necessary to modify the above apparatus and procedure. A slide holder, indicated in detail in Fig. 1, was constructed to fit into the spectrophotometer in the place of the regular absorption cell holder. Due to the fact that the sections measured were small it was necessary to make the holes a and a' so that they would mask out everything except the area on the slide covered by the section. For most of the sections $\frac{1}{4}$ inch diameter holes were used but in a few cases it was necessary to make them still smaller due to the small size of the sec-

tions available. In the original plan the unstained section was to be placed over hole *a'* and the stained section was to be placed over hole *a*; the transmittance of the stained section could then be measured relative to the unstained section by manipulating them in the same way as the two absorption cells when transmittances of solutions were being measured. This procedure was found to be impossible due to the fact that the amount of light transmitted by the two holes could not be made exactly the same. In order to overcome this difficulty all measurements were made with a plain glass slide over the hole *a*. First, a series of transmittance readings (I_O) were made for the unstained section over the range of wave-lengths desired with the unstained section over the hole *a'* by performing the series of operations for a measurement as described in the preceding paragraph, treating the piece of plain glass over the hole *a* as the cell containing the solvent and the slide carrying the unstained section over

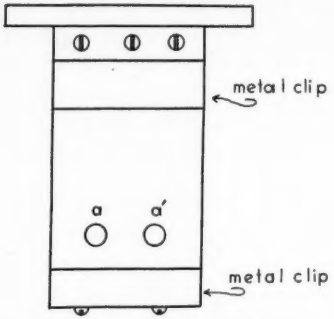

FIG. 1. Diagram of slide holder for spectrophotometric measurements.

the hole *a'* as the cell containing the solution. A corresponding set of transmittance readings (I_T) was then made with the stained section replacing the unstained section. The true transmittance was determined for each wave-length by taking the ratio I_T/I_O.

In all cases the value of $\log_{10} I_O/I_T$ was plotted as a function of the wave-length so that the absorption curves for the stained sections can be more readily compared with the absorption coefficient curves for the stain in solution. Measurements were always made on two sections of different thickness.

Some of the stain solutions (basic fuchsin, the colored product obtained in the Schiff reaction, Sudan IV and the hematoxylin) were measured on the Coleman Spectrophotometer using the method for measuring the transmittance of solutions described in the preceding section. The length of the light path in the absorption cell was 1.23

cm. and a series of dilutions of the solution were measured in order to obtain the absorption coefficients with the maximum accuracy. The rest of the spectra were measured on the recording spectroradiometer developed by Albers and Knorr at the C. F. Kettering Foundation Laboratory at Antioch College (1938). With this instrument the length of the light path in the cell was 1.00 cm. The

TABLE 2.—ABSORPTION CHARACTERISTICS OF STAINING SOLUTIONS AND STAINED TISSUES

Stain	Material	No. of measurements	Primary maximum absorption	Minimum absorption	Secondary maximum absorption
Acid fuchsin	Aqueous solution	2	540	385–395	Below 375
	Gelatin	4	550–560	390–410	Below 380
	Tendon	2	550	400–420	Below 370
	Plasma	2	550–555	420	Below 370
	Thymus	2	550–560	400–410	Below 370
Anilin blue	Aqueous solution	1	580–592	428–442	400–below 370
	Gelatin	4	580–590	430–450	Below 380
	Tendon	2	590–600	440–450	Below 370
Azocarmine G	Aqueous solution	1	515–517	430–433	392–398
	Plasma	2	530–550	440 & 380	390–400
	Thymus	2	520–560	440	400
Basic fuchsin	Aqueous solution	1	560–570	460	390–400
	Fuchsin-sulfurous reagent	1	550	—	—
	Thymus	2	550–570	400–410	Below 370
Eosin Y	Aqueous solution	1	515	—	—
	Alcoholic solution	1	525	—	—
	Gelatin	4	530	—	—
	Tendon	2	530	—	—
	Plasma	2	530	—	—
Orange G	Aqueous solution	1	478–480	—	—
	Plasma	2	480–500	—	400?
Picric acid	Aqueous solution	1	Below 370	—	—
	Plasma	2	Below 370	—	400?
Sudan IV	Alcohol-acetone solution	1	510	440–450	Below 400
	Fat	2	510	440	Below 400

values of a, the absorption coefficient, were calculated from the Beer's law formula

$$a = \frac{2.30}{cx} \log_{10} I_O/I_T$$

in which c is the concentration in grams per liter, x is the thickness of the absorption cell and I_O/I_T is the reciprocal of the transmittance. All spectral measurements were made by one of us (V.M.A.).

Results

Some of the absorption characteristics obtained in these experiments are summarized in Table 2. The maximal absorption for each curve indicates the highest reading or, in cases of two or more equal readings, it shows the range of the figures. Some stains have broad absorption bands with a gradual slope to an absorption peak which may be only slightly above the absorption at adjacent wave-lengths. Slight shifts in this maximum, which may appear misleading in the figures, account for some of the variations in figures for absorption shown in Table 2. The individual spectral absorption measurements on stained sections of both 10 and 20 μ in thickness were usually identical except for variations in height of the absorption bands. In

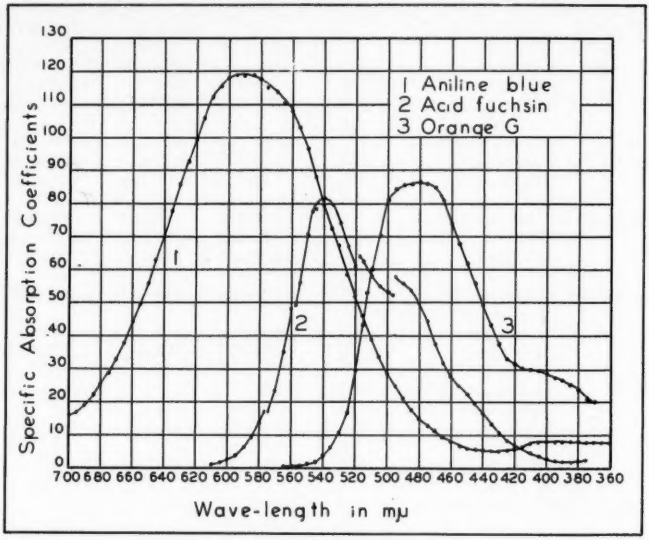

Fig. 2. Absorption curves of aqueous anilin blue and orange G acidified with acetic acid and of aqueous acid-fuchsin staining solutions. The absorption curves for the tissues stained with these dyes were similar.

a few cases showing slight variations in the curves it was necessary to increase the range of figures by 10 mμ or less to cover the outside variation between different curves on the same tissue. As an example, the 10 μ section of blood plasma stained with azocarmine G had a maximum absorption extending between 530 and 550 mμ while the maximum absorption of the similarly stained 20 μ section was 540 mμ. Hence, in Table 2 the inclusive figure 530–550 mμ is given.

Since the figures for spectral absorption maxima give scant information concerning the shape of the absorption curve, the specific absorption curves for the staining solutions and curves for some of the stained tissues are given in Fig. 2–6. Since the aqueous solutions of acid fuchsin and reduced basic fuchsin (fuchsin-sulfurous reagent) did not obey Beer's law and gave different absorption measurements with different dilutions, these curves in Fig. 2 and 4 are represented by discontinuous lines. The absorption curves (Fig. 5) of gelatin, tendon, blood plasma and thymus gland stained with acid fuchsin illustrate the maximal variations in curves of different tissues stained with the same stain. The curves of gelatin, tendon and blood plasma

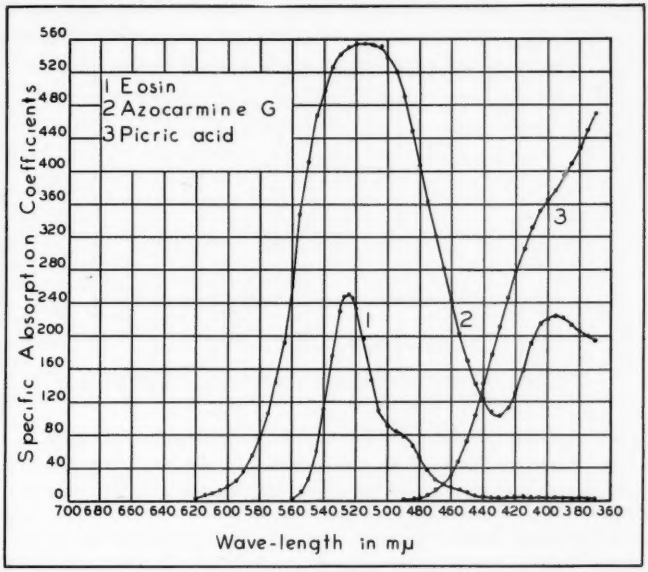

FIG. 3. Absorption curves of eosin in 95% alcohol and of aqueous azocarmine G acidified with acetic acid and of picric acid staining solutions. Similar curves were obtained for tissues stained with these dyes.

stained with eosin are strikingly similar (Fig. 6). The similarity between the absorption curves of the staining solutions and of the various tissues stained with these solutions is apparent. The curves for tissues stained with anilin blue, azocarmine G, orange G and picric acid are so similar to the curves of the staining solutions shown in Fig. 2 and 3 as to obviate the necessity of their duplication; the same statement applies to the curves of the Sudan IV stain and the fat stained with Sudan IV (Fig. 6). (Two samples of fat prepared

at different times and with different batches of dry stain gave similar absorption curves.) Sudan IV was the only stain on which our maximum of absorption bands varied greatly from that published by other workers.

The absorption spectrum of eosin was measured both in distilled water and in 95% ethyl alcohol. The curves proved very similar in shape, but differed slightly in the location of the maximum of the absorption band. The maxima of absorption in the aqueous and alcoholic solutions were located at 515 and 525 mμ respectively. (The measurements of absorption coefficients are thought to be accurate to about one per cent.)

The curve for aqueous basic fuchsin is distinctly different from that

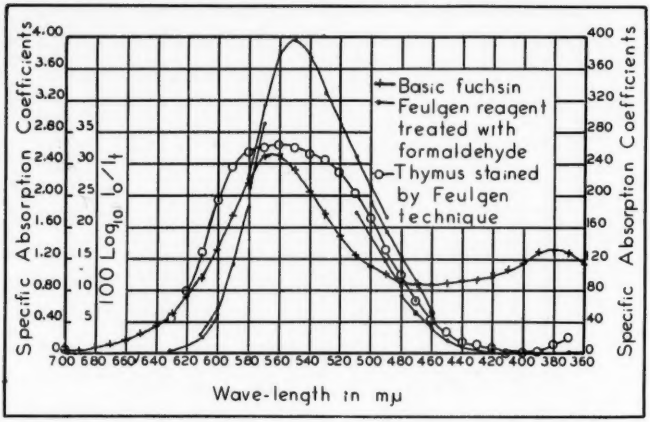

FIG. 4. Absorption curves of aqueous basic fuchsin acidified with HCl, of Feulgen (fuchsin-sulfurous) reagent treated with formaldehyde, and of thymus stained by the Feulgen technic. The scales for the specific absorption coefficients of the Feulgen reagent treated with formaldehyde and of the basic fuchsin stain are indicated on the right and left margins respectively. Thymus stained by the Feulgen technic shows a curve (with its ordinates expressed relative to 100 $\log_{10} I_O/I_T$) similar to that of the Schiff reagent treated with formaldehyde.

of the fuchsin-sulfurous solution to which formaldehyde has been added to give the Schiff color reaction; it is also noticeably different from the curve of thymus stained with the fuchsin-sulfurous solution according to the Feulgen technic. (See Fig. 4.) The last mentioned curve is much more like that of the Schiff reaction than that of aqueous basic fuchsin.

As expected, in many instances the sections 20 μ in thickness contained approximately twice as much dye as the 10 μ sections. The fact that some 10 μ sections contained at least as much dye as the

thicker sections is probably attributable to different rates of penetration of stains and destaining agents and to slight variations in technic.

Absorption measurements were made on slides with neutralized balsam and with 60% clarite in xylene which did not contain sections of tissue. Altho the absorption of light by both mounting media was slight, clarite absorbed less light. The unstained control sections of tissue which were used to determine I_0 also showed no characteristic significant absorption of visible light. The unstained human fat gave a primary absorption band at 450 mμ and a secondary band at 475.

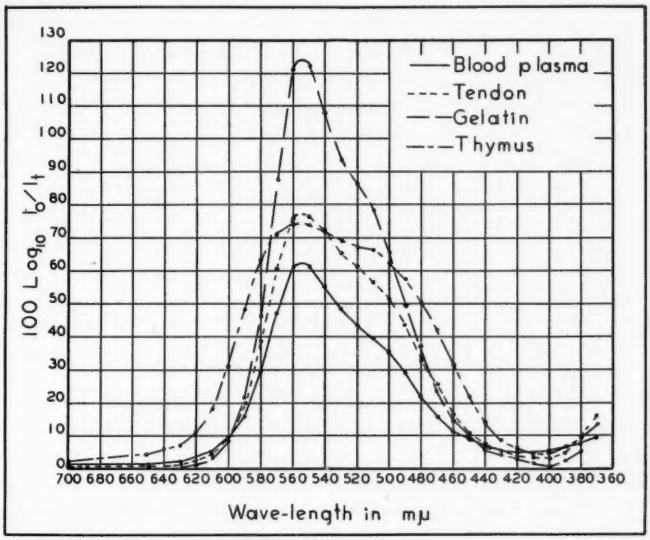

FIG. 5. Spectral absorption of 10μ sections of blood plasma, tendon, gelatin and thymus gland stained with acid fuchsin.

DISCUSSION

Numerous workers including Sheppard (1909), French (1927) and Holmes (1924) have shown that many dyes in aqueous solutions do not follow Beer's law over wide changes in concentration. Solvents also influence the absorption spectra of some dyes. For instance, benzene may produce new bands in an absorption spectrum. Probably because of decreased changes in the relative proportions of tautomeric forms of dyes, the absorption spectra of some stains can be more satisfactorily measured in alcoholic than in aqueous solu-

tions. However, except for necessary dilution, we measured the staining solutions exactly as employed with the tissues; hence, only the eosin was in alcoholic solution. Metachromatic stains were avoided because of possible difficulty with their tautomeric forms (Holmes, 1926).

Since hydrogen ion concentration, electrolytes, mordants and solvents influence the absorption spectrum of stains, one might question the feasibility of comparing the absorption spectrum of an aqueous solution with that of tissue which has been rinsed in water, alcohol, xylene and embedded in clarite. Our results, however, show that under controlled conditions the absorption spectra of the staining solutions and stained tissues are very similar. We feel—and compe-

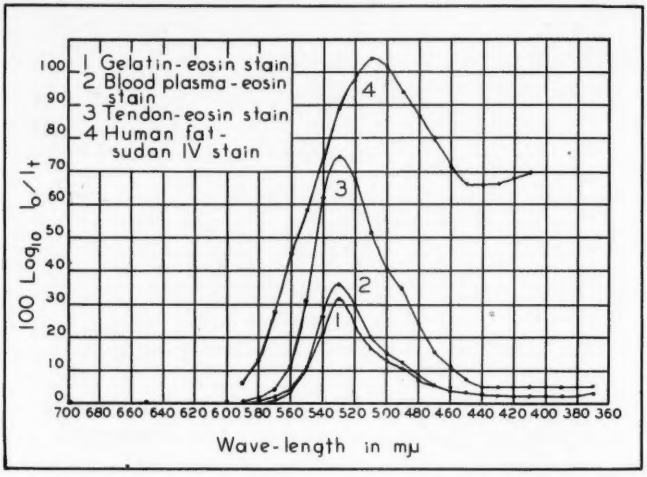
1 Gelatin-eosin stain
2 Blood plasma-eosin stain
3 Tendon-eosin stain
4 Human fat-sudan IV stain
100 Log10 b/I+
100 90 80 70 60 50 40 30 20 10 0
700 680 660 640 620 600 580 560 540 520 500 480 460 440 420 400 380 360
Wave-length in mμ

FIG. 6. Spectral absorption of 10μ sections of gelatin, blood plasma and tendon stained with alcoholic eosin solution and of human fat stained with Sudan IV. A staining solution of 1% Sudan IV in equal parts of 70% alcohol and acetone gave a curve similar to curve No. 4.

tent biologists and physical chemists agree—that the slight variations between the curves of the staining solutions and stained tissues represent technical variations and not significant changes in the chemical composition of the dye molecules. Most of the absorption curves of stains and stained tissues are as comparable as the absorption curves of the Sudan IV, which is explained on the physical basis of the fat acting as a solvent for the Sudan IV.

The comparison of the absorption curves of various types of tissue stained by the same stain shows no significant difference. Acid

fuchsin seemingly stains nuclear, cytoplasmic and connective tissue elements by a similar mechanism. Altho fixation influences the staining properties of tissues, we did not find any significant evidence of alterations in absorption curves of stains which could be attributed to different fixatives.

As definitely shown in Fig. 4, aqueous basic fuchsin represents a different dye from the fuchsin-sulfurous reagent after treatment with formaldehyde or staining of thymus. The product of the Schiff reaction has absorption coefficients 150 times those of the basic fuchsin stain, has its maximal absorption at 550 instead of 560–570 mμ, and does not show the secondary absorption band at 400 mμ. Since in the Schiff reaction and Feulgen technic the basic fuchsin is reduced by the sulfurous acid to form the fuchsin-sulfurous reagent which is further altered by the action of aldehyde groups, one would not expect the final stain to be the same as the original basic fuchsin. These differences were recognized by Feulgen and Rossenbeck (1924) who state that the final product is more blue than the basic fuchsin stain. Inspection of the curves in Fig. 4 shows that this increased blue is caused by a relatively decreased absorption of shorter wave lengths rather than by an increased absorption of the longer wave lengths. The exact chemistry of the Feulgen reaction is not understood. Wieland and Schening (1921) have suggested that aldehydes react with the colorless fuchsin-sulfurous reagent to form a colored addition compound.

The figures for the specific coefficient of absorption of the dyes are based on the dry weight of stain used in preparing the solutions. In instances in which all of the dye failed to go into solution, some error would result. The specific absorption coefficient figures for both the aqueous basic fuchsin stain and for the colored fuchsin-sulfurous reagent are based on the same initial weight of 0.5 g. of dye dissolved in 100 cc. of boiling water. Some stain was removed by the filter paper and charcoal employed in the preparation of the fuchsin-sulfurous reagent.

We do not propose to enter into the details of the controversy over the theories of staining. The chemical, solution and physical theories have been clearly stated by Holmes (1929) and Stearn and Stearn (1929) and summarized by Conn (1940, p. 188). The chemical theory proposes that the dye and tissue combine chemically to form a definite molecular reaction product. The solution or absorption theory postulates the transfer of dye molecules from the staining solution to solution within the stained substance. According to the adsorption theory, the dye is deposited on molecular surfaces by ad-

sorption. The problem is complicated by our limited knowledge of adsorption which may be either physical or chemical. Stearn and Stearn (1929, 1930, 1933) on the basis of their studies support the chemical theory of staining. Holmes (1929) gives a general summation of the evidence in favor of the physical theory of staining. On the basis of studies on effects of hydrogen ion concentration on the adsorption of dye by bacteria, Yuri (1928) and McCalla and Clark (1941) lend support to the adsorption theory. Parks and Bartlett (1935a,b) showed that the adsorption of acid and basic dyes by textile fibers was the same as the adsorption of inorganic ions by charcoal. Owens and Bensley (1929) found that osmic acid was reduced before adsorption by the Golgi apparatus. Silver (1942) presents experimental evidence and explains silver staining as an adsorption phenomenon.

The results of our experiments are readily explained on the basis of the physical adsorption or solution theories of staining. They do not exclude the possibility of minor alterations in the dye molecule which could be considered chemical. Our results indicate, however, that within the limits of the experimental methods used and for the stains employed, a chemical alteration in the chromophore group of the stain is not involved in the process of staining.

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DIFFERENTIATING ABNORMAL CELLS IN VALENCIA ORANGE VESICLES

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Abstract.—**Metamorphosing cells developing thick cell walls in the juice vesicle of granulated Valencia oranges, which cannot be detected by ordinary microscopical examination, may be differentiated in an early stage for microchemical analysis in lignification studies. Thick sections of the juice vesicle are treated overnight in a 1:10,000 aqueous solution of ruthenium red, followed by 5–10 minutes in a 1:5,000 aqueous solution of methylene blue, and then differentiated in water.**

The Valencia orange ripening in the summer months in southern California is subject to a disorder called granulation.[1] It is not caused by a pathogen but is apparently physiological in nature. The sap gels in the cells of the juice vesicle, then slowly disintegrates in certain cells whose walls may thicken to as much as 25 times normal. This transformation is accompanied by the production of bubbles of gas which accumulate in a central cavity formed in the vesicle by the splitting of the adjacent walls of the metamorphosing cells at the middle lamellae. The apparent similarity between granulation in Valencia orange fruit to the little understood process of lignification in the secondary structure of plants in general, suggests the importance of selection of cells in initial stages of granulation in portraying the micro-chemical changes occurring in early stages of lignification.

The cells which will undergo the aforesaid changes and which cannot be identified by ordinary microscopical examination, may be distinguished thru staining reactions at an early stage for chemical and physical characteristics. Median longitudinal free hand sections 300μ to 400μ in thickness (in transverse section, isodiametric cells on interior of sac are 200μ or more in diameter) are made of the hardening juice vesicle before gas bubbles are evident. The sections are placed in a 1:10,000 aqueous solution of ruthenium red—$Ru_2(OH)_2Cl_4 \cdot 7(NH_3) \cdot 3H_2O$—for 16 to 18 hours. The cell walls of normal

[1]Bartholomew, E. T., Sinclair, W. B., and Turrell, F. M. 1941. Granulation of Valencia oranges. Univ. of Calif. Expr. Sta. Bul. 647, 63 pages.

cells contain considerable pectin which absorbs ruthenium red readily. They stain a rather deep red, the depth of color depending on the length of time in the stain. If gas bubbles are visible in the unsectioned vesicle, thick-walled cells are present and these cell walls will not stain with ruthenium red, indicating pectin has been transformed or withdrawn from them.

The sections are washed with distilled water and then placed in a 1:5,000 aqueous solution of zinc-free methylene blue[2] for 5 minutes, transferred to distilled water and washed for 2 minutes, transferred to fresh distilled water and washed from 5 to 10 minutes. Cells which have thickened cell walls or which would develop thick cell walls stain blue.

Differentiation of the two stains in distilled water can be followed under a dissecting microscope. Excess blue is abstracted from the cells which stained with ruthenium red, but water does not destain

FIG. 1

FIG. 2

Photomicrographs of a longitudinal section through a Valencia orange juice vesicle in the early second stage of granulation, stained with ruthenium red and methylene blue.

FIG. 1. Section photographed with a red filter showing methylene-blue-stained cells which will develop into thick-walled cells.

FIG. 2. Same section as Fig. 1, but photographed with a red filter to show the developing gas bubble in center of vesicle and general cellular structure of the juice vesicle composed of normal cells whose walls stain with ruthenium red.

appreciably the cell walls which stain blue heavily. These cell walls in advanced stages are probably lignified[3] and stain blue with methylene blue similar to the lignified tissue in wood. Metamorphosing

[2]Coleman and Bell's "methylene blue, for bacilli" used in this work.

[3]Turrell, F. M., and Bartholomew, E. T. 1939. Structural and microchemical changes in granulated orange vesicles. Calif. Citrog., 24, 88, 110.

cells differentiated from the normal cells of the juice vesicle are shown in figures 1 and 2.

Cells which have not undergone lignification but which are susceptible to such change accumulate methylene blue in the oxidized form. It is also notable that vesicles which are undergoing changes in the granulation process have higher oxidation-reduction potentials than normal vesicles.

A NISSL METHOD USING BUFFERED SOLUTIONS OF THIONIN[1]

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ABSTRACT.—**Experiments were performed in an attempt to obtain a rapid method for staining the chromophilic substance of formalin-fixed nerve cells differentially without resorting to over-staining and subsequent acid-decolorizing. A satisfactory procedure using thionin in dilute buffered solutions was developed: Prepare a stock solution of the dye using 1 g. in 100 ml. of distilled water. Prepare veronal-acetate buffers (Michaelis, 1931) in the range of pH 5 to pH 3.5. To each 10 ml. of the buffer add 0.5 ml. of the stock dye solution. After rinsing in CO_2-free distilled water place mounted or unmounted sections in this solution. (Freshly fixed material, 10μ to 20μ thick, is completely stained in 10 to 20 minutes but over-staining does not occur when longer times are allowed.) Return sections to distilled water (2 changes) and wash until diffusion of excess dye is no longer visible. Wash in 70% ethyl alcohol (2 changes) until diffusion of excess dye is no longer visible. Dehydrate in 95% ethyl alcohol and normal butyl alcohol, clear and mount.**

Optimum staining of chromophilic material occurs at pH 3.65. Glial processes are well stained at pH 4.6. Nissl bodies and glial cytoplasm are purplish blue, nuclear chromatin is blue, background is clear at pH 3.65 but pale blue at pH 4.9.

Staining of the chromophilic substance of nerve cells can be accomplished by many procedures using various basic dyes. The original methods of Nissl (1891; 1894) and many of their numerous modifications (Lee, 1937) are cumbersome and relatively unreliable. Other procedures, such as the gallocyanin, gallamin-blue, and anthracene-blue technics of Einarson (1932; 1935), Einarson and Bentson (1939), are time consuming. Many require acid decolorization after staining (Tress and Tress, 1935; Neumann, 1942; Dei Poli and Pomerri, 1938). This is often undesirable because the tigroid bodies of some nerve cells give up dyes more quickly than in others, and it is seldom possible to accomplish differentiation evenly. In general, most staining technics which are in common use would seem to have

[1]Aided by a grant from the Abbott Foundation of Northwestern University.

been derived more or less accidentally and are more suggestive of recipes from the kitchen cook-book than of scientific procedures.

It has been widely recognized that the chromophilic substance can be displayed with greater clarity after fixation of tissues in formaldehyde solutions or alcoholic fluids containing a small amount of weak acid than in certain other histologic fixing fluids, such as Zenker's mixture. Hopkins (1924) and Sheinin (1932) have studied the effects of different fixatives and concluded that the appearance of Nissl bodies depends on the substances used. It is therefore important to control the fixation in any experimental series. Sheinin (1932) reported that the pH of fixing fluids containing metallic salts, within limits on the acid side, is not the determining factor in altering the morphology of the chromophilic material. On the other hand, Shibata (1933) found that variations in the pH of formalin (1:10) used as a fixative did result in variations in stainability and size of the Nissl bodies. The optimum pH of the fixing fluid for subsequent staining with toluidine blue appears to have been somewhere between 5 and 4. This observation is of special interest in the light of our present results.

Few investigators except Marshall and Einarson and co-workers appear to have attempted to control staining of the chromophilic substance of nerve cells by buffering and adjusting the pH of the staining bath. Marshall (1940) used a pH of 5.9 (calculated) but the dye was precipitated by the buffer. Einarson and Ringsted (1938) and Einarson and Bentson (1939) altered the pH of gallocyanin solution within the range of 1.24 to 3.27 and concluded that the specific staining of Nissl bodies was not influenced altho the nonspecific costaining of the cytoplasm was affected. A few years ago Davenport, working in this laboratory, obtained some successful preparations by bulk-staining of formalin-fixed spinal cord tissues in a dilute buffered solution of safranin at about pH 4. He did not publish his results but one of us used this procedure in a study of nerve cells in ventral roots (Windle, 1931). Other investigators have introduced an uncontrolled amount of acid to the dye bath to improve the staining of sections with fuchsin (Dei Poli and Pomerri, 1938), thionin (King, 1910; Bean, 1927) and neutral red (Kirkman, 1932). Johnston's (1916) and Morgan's (1926) procedures with ripened neutral red depend upon development of acidity for their success. It is clear that differential staining was enhanced by keeping the solution on the acid side of pH 7. With compound blood and tissue stains such as Wright's or Giemsa's preparations, French (1930) used buffered solutions (pH 5.3 to 9.2) in preliminary treat-

ment, in staining or in differentiation and found that H-ion control was essential to consistent results.

Our problem has been to obtain complete and very constant staining of the chromophilic material of nerve cells in sectioned brain and spinal cord material fixed in formalin. We wished to simplify and increase the dependability of the Nissl method in order to compare tissues from experimental and control animals critically. Altho a high degree of selectivity was desired, methods employing differentiation in anilin-alcohol or acid-alcohol introduced undesirable factors of variability which it was hoped to avoid. We wished if possible to stain the neuroglia to a limited degree as well as the neurons. The following experiments were performed and a method utilizing thionin as the dye was developed.

Materials and Methods

Three lots of formalin-fixed tissue were used, namely, spinal cord of a newborn guinea pig, spinal cords of young adult guinea pigs, and adult human spinal cord. The guinea pigs were perfused with the fixing fluid after killing with nembutal. The human cord was removed 9 hours after death. The newborn and human spinal cords had been preserved in formalin (1:10) and in blocks of nitrocellulose in 70% ethyl alcohol for periods of several months and eleven years, respectively. Other young adult guinea pigs were perfused with Carnoy's, Zenker's, and Bouin's fluids.

Material from all sources was embedded in low viscosity nitrocellulose and sections were cut 20μ to 50μ thick. Some of the specimens from the young adult guinea pigs were embedded in paraffin and sectioned at 10μ. Paraffin sections were mounted on glass cover slips which were subsequently handled like celloidin sections. We used a few celloidin sections of human spinal cord which had been cut 9 years previously and stored in alcohol as well as some sections of guinea pig brains which had been stored between squares of thin paper for several weeks.

The dyes to be tested were prepared by dissolving one gram of powder in 100 ml. of distilled water. These stock solutions were filtered once and diluted before using. The following dyes were selected:

Toluidine blue (National Aniline, Certification No. NU-1)
Cresyl violet (National Aniline, Certification No. NW-6)
Cresyl echt violet (Coleman & Bell)
Methylene blue chloride (National Aniline, Certification No. NA-11)
Hematein crystals (Coleman & Bell)

Safranin bluish, *Syn.* methylene violet, C.I. No. 842 (National Aniline)

Thionin pur. (Grübler)

Thionin (National Aniline, Certification No. NT-9)

Staining was carried out in solutions made up of 0.25 or 0.5 ml. of the stock dye solution added to 10 ml. of a veronal-acetate buffer (Michaelis, 1931). A phosphate buffer was tried but proved unsatisfactory because the stain precipitated in it. Similar results were reported by Marshall (1940). The Michaelis buffer was prepared as follows:

Solution A:

$NaC_2H_3O_2+3H_2O$	9.714 g.
Veronal-sodium	14.714 g.
Distilled water (CO_2-free), *to make*	500 ml.

Solution B:

NaCl, C. P.	42.5 g.
Distilled water (CO_2-free), *to make*	500 ml.

Solution A is 1/7 molar in respect to the sodium acetate as well as the veronal-sodium. To each 5 ml. of Solution A were added 2 ml. of Solution B, a variable quantity (*a* ml.) of N/10 HCl and 18 minus *a* ml. of CO_2-free distilled water. Relationships between the quantity *a* and the pH can be obtained from Michaelis' (1931) article. We first prepared the whole range of buffers but finally eliminated those which were of no use. The following are the most useful:

a ml.	18-*a* ml.	pH
8	10	5.32
10	8	4.66
12	6	4.13
14	4	3.62
16	2	2.62

The actual pH of each buffer was determined with the glass electrode. Wide variation between these and the stated pH values was found only in the lowest range.

In all experiments the following steps were taken in staining and mounting sections: From 70% alcohol sections were placed directly, or after rinsing in distilled water, into the buffered dilute dye solution. After staining they were transferred to distilled water (2 changes) where they remained until diffusion of excess dye from them was no longer visible. They were then passed thru two changes of 70% ethyl alcohol; diffusion of excess dye from tissue and nitrocellulose was observed (most color came out in the water) and when

no longer visible the sections were dehydrated in 95% ethyl alcohol and normal butyl alcohol, cleared in xylene and mounted in "clarite." All distilled water was rendered CO_2-free by boiling. Fluids were changed at frequent intervals. Staining was carried out at room temperatures averaging about 75° F.

Results

Fixing Fluids.—Both Carnoy's fluid and formalin (1:10) proved to be excellent for preservation and subsequent staining of the chromophilic material of nerve cells. Bouin's fluid we rated a poor third choice because less sharp differentiation of Nissl bodies was obtained after using it. The material fixed in Zenker's fluid was of no value for our present purpose because the Nissl bodies failed almost entirely to hold the stains. It seemed that they had been rendered unstainable by some component of the fixing solution. The results with Carnoy's fluid were a little more pleasing than those with formalin, but the difference was not great.

In respect to structures other than the Nissl bodies of nerve cells, formalin was the best fixing fluid. The glial cytoplasm was more clearly depicted after formalin than after Carnoy's fluid. Because the present experiments were devised in an effort to find a suitable method for use with formalin-fixed material, less attention was paid to the three other fixatives.

The descriptions which follow apply to the guinea pig spinal cord unless otherwise stated.

Toluidine Blue.—Solutions containing 0.25 ml. of the stock dye solution and 10 ml. of buffer (pH 8.49 to 2.1) were used.

Staining in all buffers down to pH 3.65 was as complete as desired in 20 to 30 minutes but the end-point was not sharp and over-staining occurred in an hour. The best results were obtained in buffers of pH 4.9 to 4.4; below these the chromophilic material took up the dye too weakly. However, the background retained a blue color at all values down to pH 3.65 and this detracted from the final appearance of the preparations.

Neuroglia stained well with toluidine blue at pH 4.9. The cytoplasmic processes of astrocytes, oligodendrocytes and microglia could be clearly distinguished. All elements in the sections were colored blue, however, and little metachromatic effect was obtained. In general, toluidine blue was found to be inferior to some of the other dyes tested for our purpose.

Cresyl Violet.—Solutions of two lots of the dye were prepared as in the case of toluidine blue. The buffers used ranged from pH 7.9 to 2.29.

The cresyl violet obtained from the National Aniline is entirely different from that put out by Coleman and Bell. It is a blue dye which precipitated in about 10 minutes when added to the buffers. Cells became weakly blue in that time.

The other lot of dye (Coleman and Bell) gave a violet color to the Nissl bodies and the glial cytoplasm at pH 5.2; nuclei were bluish and the background "reticulum" was pale blue after an hour's time. Results were good, but the nitrocellulose retained more color (pink) than by other methods. At pH values below 4.9 the nerve cells were too light and at pH 5.8 they were too dark. Glia were not well stained.

When the concentration of dye was increased to 0.5 ml. in 10 ml. of the buffers, complete staining was obtained in 45 minutes. By prolonging the time greatly (15 hours) we could bring about overstaining. The most pleasing results were had at pH 4.9 but the coloring of the nitrocellulose and background made cresyl violet of lesser value than thionin.

Methylene Blue Chloride.—The dye was used in the concentrations and at the pH values employed for toluidine blue. Best results followed staining at pH 4.6 to 4.4 but there was no range in which the background remained clear while the cells stained adequately. An end-point was reached within 30 minutes.

Hematein.—The dye baths were prepared by adding 0.5 ml. of the stock solution to buffers from pH 9.54 to 2.1. Hematein acted as an indicator, solutions turning from red to yellow between pH 7.65 and 4.9.

The Nissl bodies of nerve cells were only faintly stained above pH 5.8 and below pH 9.0. They remained unstained in the other buffers. Nuclear chromatin stained well above pH 4.6. Maximum staining was attained within 30 minutes.

Safranin Bluish (methylene violet, water soluble, C.I. No. 842).—The dye baths were prepared as for other stains. It was found that usable results were obtainable only in the range of pH values below pH 4.4. Even at pH 2.1 the background retained color, altho Nissl bodies were too lightly stained.

Results were disappointing in view of Davenport's and our former success with bulk-staining. However, a Grübler dye (probably safranin O, C.I. No. 841) was used at that time and we have no record by which to identify it. Furthermore, at that time we were unable to stain sections satisfactorily with this dye.

Thionin.—Two lots of dye were used and no significant difference between them was noted. We used 0.5 ml. of the stock solutions and 10 ml. of buffers from pH 7.9 to 2.1.

The optimum staining time for material fixed in Carnoy's fluid was found to be 20 minutes. For fresh formalin-fixed guinea pig spinal cords staining was as complete in 10 minutes as it was at 20 minutes, but the material which had been stored for several months required 15 to 20 minutes to reach an end-point. The human spinal cord sections (11-year-old material) required an hour to stain. Some 50μ sections which had been stored for years between numbered slips of paper in 70% alcohol failed to become fully stained in less than an overnight period. A rather definite end-point was reached quickly in case of the freshly fixed material. Over-staining was not obtained by leaving sections in the dye solutions overnight.

The best picture of nerve cells and their chromophilic material resulted from staining at pH 3.65. At pH 5.2 the cytoplasmic background and the extracellular background retained color. At pH 2.68 Nissl bodies were incompletely stained. A metachromatic effect was obtained, especially with Carnoy-fixed spinal cord, at pH 3.65; Nissl bodies were purplish blue, nuclear chromatin was blue, background and nitrocellulose retained almost no color.

Neuroglia were inadequately stained at pH 3.65, but they were brought out in a striking manner in the guinea pig material at pH 4.6 and even at 4.4 they were well stained. The cytoplasmic processes of all three types took on a purplish blue color, while the nuclei appeared lighter blue. In the old human material the glia failed to stain adequately. Some sections of guinea-pig brains which had been stored in alcohol between slips of paper for several weeks stained slowly and the glial cytoplasm failed to take up the dye. It would seem that glia can be clearly distinguished only in reasonably freshly fixed and freshly sectioned material. Formalin proved superior to Carnoy's fluid for this purpose but had less of a metachromatic effect.

Determining the optimum pH at which to stain spinal cord material involves a factor of personal preference. For some purposes it may be desirable to use buffers with slightly higher or lower pH values than those we have reported as optimum. The large multipolar neurons of the spinal cord are nicely stained at pH values too low for adequate coloring of smaller neurons of the brain.

Summary of Recommended Method

Fixation recommended: Carnoy's fluid or formalin (1:10). The latter is preferable when glial processes are desired; prolonged fixation is undesirable for glia and increases staining time for neurons.

Embedded in: Low viscosity nitrocellulose or in paraffin. Thick sections require a little longer time in staining and other solutions.

Preparation of solutions:

I. The buffers: (See page 64)

II. The staining solutions:

Stock solution—filter after dissolving dye:

Thionin	1 g.
Distilled water	100 ml.

Dilute staining solutions:

Buffer of chosen pH	10.0 ml.
Stock staining solution	0.5 ml.

Procedure:

1. Place nitrocellulose sections or mounted paraffin sections (after removal of paraffin) into distilled water (CO_2-free).
2. Transfer to the buffered staining bath.
   a. For Nissl bodies of large motor neurons approximately pH 3.5 (range pH 4.5 to pH 3) is recommended.
   b. For cytoplasmic processes of glia (formalin-fixation) approximately pH 4.5 (range pH 5 to pH 4) is recommended.

   Stain at room temperature for 10 minutes or more. For old material or thick sections longer periods will be required. Staining reaches a relative end-point; over-staining does not occur.
3. Transfer to distilled water (2 changes) and allow excess dye to diffuse out of sections.
4. When its diffusion is no longer visible (about 2–3 minutes), transfer to 70% ethyl alcohol (2 changes). Allow sections to remain in alcohol until diffusion of dye from them is no longer visible (about 2–3 minutes).
5. Transfer to 95% ethyl alcohol (for about one minute).
6. Clear and mount.

Results:

In lower pH range:

Nuclear chromatin	blue
Chromophilic material	purplish blue
Glial cytoplasm	only faintly seen
Background	clear

In higher pH range:

Nuclear chromatin	blue
Chromophilic material	darker purplish blue
Cytoplasmic background of nerve cells	not clear

Glial cytoplasmic process.....purplish blue
Background and nerve fibers.....light blue

Note: Better metachromatic effects followed Carnoy-fixation than formalin.

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THE HORTEGA SILVER CARBONATE METHOD FOR STAINING PITUICYTES AFTER PARAFFIN EMBEDDING

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In three papers published on pituicytes, of the ox by Bucy (1930), of the human by Shanklin (1940), and of the horse by Vazquez-Lopez (1942) the pituitaries were sectioned by the freezing method and stained by the Hortega silver carbonate technic. Since that time, as a routine procedure in our laboratory, frozen sections have been replaced by paraffin which in no way interferes with the Hortega silver carbonate staining.

Paraffin sections have many advantages over frozen sections that are known to all histologists. Sectioning after paraffin is far more rapid, the sections are of more uniform thickness, and no sections are lost. By handling the sections in ribbons, they are mounted more rapidly and more uniformly on the slide. The majority of the material in the writer's laboratory is mounted as serial sections. This enables the study of variations in the constituents of different parts of the neurohypophysis and the adjacent part of the hypothalamus. These sections also make it possible to use the material for modeling. In the preparation of the human material, referred to above, and also that of the adult dog, each section after cutting on the freezing microtome is handled separately thru each stage: washing, impregnating, reducing, toning, dehydrating, clearing and mounting. Each section is handled 14 times. When cut at 12 μ the pituitary of an adult dog makes about 400 sections; if each section is handled 14 times that makes 5600 operations.

Far more serious than the time consumed, in the case of the freezing method, is the damaging and loss of sections, because many sections become folded and broken. In fact, many are broken so badly they have to be discarded, and still more are partially damaged. In order to overcome the above objections to the freezing method, experiments with the use of paraffin were begun on learning that Foot (1924) and Foot and Menard (1927) had successfully applied the Hortega silver carbonate method, following paraffin imbedding, to the study of reticular fibers.

The Hortega silver carbonate technic, as used in the writer's laboratory, follows closely the description by Penfield and Cone (1937), especially as used for staining microglia and oligodendroglia.

Naturally the ideal method is to place the pituitary in the fixative immediately after removal. However, that is not always feasible when using human material subject to autopsy. It would seem that pituicytes are rather stable since successful staining has been obtained on one human fetus after 60 hours of refrigeration. The entire pituitary of nearly all animals can be well fixed; however, when large pituitaries, such as the ox, are used, part of the pars anterior should first be removed.

Fixation has been carried out in Cajal's formalin-ammonium-bromide solution as recommended by Penfield and Cone (1937). They advise the use of Merck's blue label 40% formalin. This brand has proved highly satisfactory, but other brands purchased here in Beirut or on hand in the various laboratories of this university have not given satisfactory results, altho they were in constant use for other kinds of histological or pathological technic. A liberal amount of the fixative should be used.

Fixation usually requires three days at room temperature, but in winter that may be insufficient. During cold weather we place the tissue in an incubator between 30° and 35° C.

The method is outlined as applied in this laboratory:

1. Fix entire pituitary in Cajal's formalin-ammonium-bromide[1] for three days at room temperature.
2. Wash 2 to 3 hours in redistilled water to which 15 drops of concentrated NH_4OH per 100 cc. have been added to remove formalin. Keep container covered.
3. Wash for one hour in 100 cc. redistilled water.
4. Dehydrate with alcohol, 70%, 80%, 95%, leaving the sections in each 3 hours and in the absolute alcohol 4 to 6 hours.
5. Clear in cedar oil overnight, followed by xylene for one hour.
6. Infiltrate with paraffin (melting point 48° to 50°C.) from 3 to 4 hours and embed in paraffin (melting point 62°C. in summer and 50 to 52°C. in winter).
7. Cut sections 8 to 12 μ on a Spencer rotary microtome.
8. Mount on slides with egg albumen and leave to dry thoroly, at least 24 hours if at room temperature.
9. Remove paraffin by passing thru two changes of xylene, followed by absolute alcohol, 95% and 80%.
10. Wash in distilled water.
11. Wash twice in redistilled water one or two minutes each time.
12. Transfer into strong $AgNO_3$ solution[1] and heat for a few minutes

[1]For this and other formulas see Penfield and Cone (1937, pp. 519, 520).

at about 50°C.[2] As a rule three minutes is sufficient, however the time is varied until the optimum is determined.

13. Place in redistilled water for one minute; do not agitate.
14. Reduce in 1% neutral formalin in redistilled water for one minute, agitating sections gently.
15. Wash in redistilled water for one minute.
16. Tone in yellow gold chloride (1 g. in 500 cc. redistilled water) until the last trace of yellow or brown is replaced by gray.
17. Place in Cajal's fixing bath[1] ($Na_2S_2O_3$) about one minute.
18. Wash in distilled water.
19. Dehydrate in alcohol and clear in xylene.
20. Mount in neutral Canada balsam and cover with cover slip.

Pituicytes have been successfully stained by the Hortega silver carbonate method after paraffin embedding in the human, dog, cat, pig, jackal and chick. For some undetermined reason the technic has consistently failed in the case of pituicytes in the mouse, rat and rabbit. In a recent paper, Vazquez-Lopez (1942) reports favorable results on rabbit material with Cajal's gold-sublimate method. In addition to pituicytes this method also stains well the ependyma, nerve cells, and connective tissue, especially the reticular fibers. Apparently paraffin sections can also be used in the study of microglia and oligodendroglia when stained by the Hortega silver carbonate method.[3]

The writer wishes to express his thanks to Mr. Tamir Nassar for helping to solve this technical problem.

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[2]The solution is usually good for two slides with about 30 sections each.

[3]The application of the Hortega silver carbonate method following paraffin sections was made independently by the writer who was unaware that a similar application had been made at an earlier date by Griffiths, M., Proc. Linn. Soc. N.S.W. **63**, 81, 1938; and Endocrinology **26**, 1032, 1940.

THE ACTINOMYCES OF POTATO SCAB DEMONSTRATED BY FLUORESCENCE MICROSCOPY

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ABSTRACT.—**The potato scab *Actinomyces*, like other acid-fast organisms, can be selectively impregnated with carbol-auramin and when exposed to ultraviolet radiation fluoresces bright yellow. The marked contrast of the bright yellow filaments permits ready localization and study of the micropathology of the tissue with a simple fluorescence microscope. The staining technic is done at room temperature and no counterstain is used. The fluorescence technic confirms Lutman's conclusion that the filaments are intercellular and grow within the middle lamellae. After complete removal of the paraffin, the sections are stained 4 minutes in carbol-auramin, (distilled water 97 ml., liquified phenol 3 ml., certified auramin 0.1 g.), washed, destained in a 0.5% solution of NaCl in 70% alcohol with 0.5 ml. HCl (conc.) per 100 ml., washed, and mounted in glycerin.**

Fluorescence is the property of certain substances whereby invisible ultraviolet radiation is absorbed and emitted as visible light. Since acid-fast organisms absorb and retain certain fluorescent acid-fast materials in the same way as they do acid-fast dyes, the principle affords a new approach to the problem of demonstrating such organisms. In practice, it appears to have certain advantages over other methods.

The organisms are impregnated with carbol-auramin or carbol-thioflavine which are removed by special acid alcohol from all except the acid-fast material. When irradiated with long-wave-length ultraviolet radiation, the auramin fluoresces bright yellow. The contrast between the bright yellow organisms and the dark background permits study at lower magnifications than possible with the usual staining methods. The method is made available by a simple high intensity concentrated filament lamp with a suitable ultraviolet transmitting filter. A complimentary yellow filter, placed in the eyepiece, gives a dark field by preventing any blue light reaching the eye (Richards and Miller, 1941). With tuberculosis bacteria the fluorescence technic has been found more sensitive than the usual Ziehl-Neelsen staining (Richards, Kline, and Leach, 1941).

The potato scab *Actinomyces* has been shown by Lutman (1941) and others to be Gram-positive. The ordinary staining methods

proved inadequate and Hutchins and Lutman (1941) devised a special modified Gram stain for this purpose. This suggested that the fluorescence technic might be simpler and more direct. Professor Lutman kindly sent me some small potato tubers embedded in paraffin and some slides stained with their method. Sections were cut at 10μ and fastened to the slide with a minimum amount of Mayer's albumen fixative. Considerable care was taken to remove the paraffin with two changes of fresh xylene, because paraffin itself is fluorescent. The sections were passed thru graded alcohols to water and stained for 4 minutes in carbol-auramin [distilled water, 97 ml.; liquefied phenol, 3 ml.; auramin O (C.I. 655),[1] 0.1 g.] The slides were then washed for 2 minutes under running tap water. Most of the auramin was washed out with a small amount of the destaining solution (70% alcohol, 100 ml.; concentrated HCl, 0.5 ml.; NaCl, 0.5 g.) Further decolorizing was done in two separate batches of the decolorizing solution for approximately 3 minutes each. The slides were washed, mounted in glycerin, and in order to make semi-permanent preparations, ringed with gold size. All procedures were carried out at room temperature and no counterstain was used.

The dye dissolves slowly with shaking or may be dissolved rapidly with gentle heat. Upon cooling the stain may become cloudy. Cloudy solutions are entirely satisfactory, but if much reddish material appears on the surface it is well to filter the stain thru ordinary filter paper before using. Some investigators dissolve the auramin in 10 ml. alcohol; adding it to the dilute (3%) phenol gives a solution which remains clear for a longer period of time. The stain will keep for several months but should be discarded as soon as the fluorescence appears less bright. It may be desirable to check the stain by staining a known acid-fast organism.

The *Actinomyces* hyphae are seen as bright yellow filaments on a nearly dark background. The cell walls do not stain (Fig. 1A and B). A few minutes may be required to see the fluorescence when the eye has to adapt from bright light to the less intense fluorescence. Maximum visibility occurs when extraneous light is prevented from reaching the mirror or the preparation on the microscope stage and may be achieved by placing the microscope in a semi-dark room, in a corner away from windows and bright lights, or by building a shield of black cloth or other material around the microscope.

The filaments are easily seen with a 16 mm. objective and a 10× eyepiece. The 8 mm. objective with a 20× eyepiece gives better

[1]That certified by the Commission on Standardization of Biological Stains is recommended.

contrast and detail at the same magnification as the 4 mm. objective and, in addition, provides greater working distance and depth of focus.

Carbol-thioflavine (C.I. 815) has a slightly brighter fluorescence and may also be used in this technic; longer decolorizing may be necessary. The usual mounting materials, such as balsam, damar and clarite fluoresce and are unsuited for this purpose. Old preparations may lose their fluorescence, but may be restained.

The filaments are intercellular and may be traced between the cells and their branching followed. This does not show so well in the photomicrographs, because of the limited depth of focus, but under

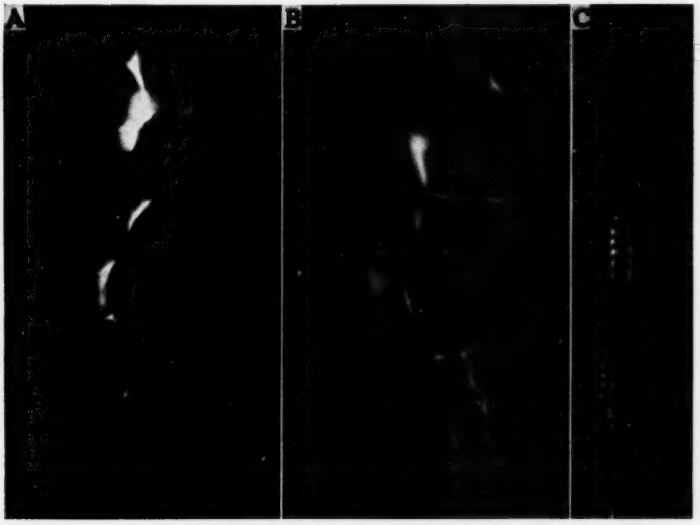

FIG. 1. A and B. Photomicrographs of *Actinomyces* filaments in a potato tuber. C. Acid-fast spiral vessel. 300×.

the microscope they may be followed within the limits of the section. The bright masses are excretions from the fungus which are also acid-fast.

The only acid-fast material found in the potato tuber sections besides the fungus was a spiral vessel, Fig. 1 C. The size and form of the vessel as compared to the fungus elements is such that no confusion is likely to result from their both being acid-fast. A few of these were seen, mostly toward the center of the tuber section and they may have been carried in by the knife as the section was cut.

Lutman believes that the filaments of the Actinomyces grow between the cells but other observers hold that he stained the middle lamella itself. By middle lamella is to be understood in the latter case, the structures observed and named by Mangin, composed of calcium pectate, and not the pectin matrix in which they are imbedded. This matrix is not acid-fast or Gram positive. The ends of these filaments, however, stop abruptly in many places and do not circumscribe the cells, only one or two walls showing them while they are absent on the others. Consequently, the fluorescent method is in agreement with and confirms Lutman's conclusions.

The chief advantage of the fluorescence technic for revealing *Actinomyces scabies* is that only the filaments and their excretions are sharply stained. If infection is present, it is very readily found. It is possible to follow the filaments and determine the extent of the fungus growth. The marked contrast of the bright yellow filaments against the dark background allows them to be followed with objectives having less magnification and greater depth of focus. The procedure is simple, it is not necessary to hold strictly to the schedules given, and the concentrations of the stain and destaining solution may be varied somewhat and still be effective. The procedures are easily carried on at room temperature and no counterstain is required.

The fluorescence technic could probably be applied to other fluorescent organisms such as the ring-rot fungus described by Ivorsen and Kelley (1940). It would have no advantage over their equipment for selecting seed stock, but might be advantageous in studying the pathology of the invaded tissues.

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A QUADRUPLE TISSUE STAIN FOR STRONG COLOR CONTRASTS

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With the increasing use of Kodachrome photomicrographs for revealing the finer structure of organs, it seemed useful to develop a staining method to bring out the tissue elements in strong contrasting colors. As four dyes are used, the method has come to be called the "Quad" stain, in the author's laboratory. The method has been used for two years on quite a variety of tissues, most of which have yielded excellent color films for teaching purposes. The slides have not faded. During this period two satisfactory new dyes have been obtained to supplant imported products originally used, namely, synthetic orcein of MacAndrews and Forbes and National Aniline acid alizarin blue 2B.

In devising this "Quad" stain we have borrowed freely from the methods originated by: Mallory (1900), Petersen (1924) and Crossmon (1937). Many structures ordinarily difficult to demonstrate are clearly defined by this method. These include basement membranes, cell boundaries and neuro-fibrils.

PREPARATION OF SOLUTIONS

Solution 1. Orcein, synthetic, 0.4 g. (MacAndrews and Forbes product, Cert. No. FOr2, used); HNO_3 C.P. conc., 0.4 ml.; 90% ethyl alcohol, 100 ml.

Solution 2. Dissolve 0.35 g. acid alizarin blue (National Aniline; lot 9854 used) in 100 ml. 10% aqueous $Al_2(SO_4)_3+18H_2O$. Boil gently 10 minutes; restore evaporated water and add six drops of strong aqueous $FeCl_3$ (40 g. $FeCl_3$ to 60 ml. H_2O). The iron has two effects: it makes a clearer nuclear stain, and makes the stain bluer. If more reddish tones are desired, the amount of iron may be decreased.

Solution 3. Orange G., dye content 87%, 2.0 g. (National Aniline batch, Cert. No. NO-6, used); fast green FCF, dye content 93%, 0.2 g. (National Aniline NGf-3, used); glacial acetic acid 2.0 ml.; distilled water, 100 ml.

All three solutions are quite permanent. Solution 1 tends to stain more rapidly after it is several days old, when the staining period should be shortened. Solution 1 is best used as a progressive stain.

METHOD

1. Fix in Zenker or Helly's solution.

2. Embed in paraffin, nitrocellulose or parlodion and section 10 μ or less.

3a. Affix nitrocellulose or parlodion sections to the slide as follows: Float section from 95% alcohol onto clean slides and straighten out with fine brush or glass rod. Blot firmly with smooth, hard blotting paper or bibulous paper and immediately let several drops (the fewer the better) of 0.25% paralodion in ether-alcohol, flow down the slide beyond the section. Look across the film with slide at an acute angle and when the surface begins to look dull or slightly wrinkled place immediately in 85% alcohol to harden the parlodion (about 30 minutes).

3b. Mount paraffin sections on slides as usual and pass thru xylene and absolute alcohol down to 85% alcohol.

4. Remove any $HgCl_2$ in the sections with iodine in 85% alcohol.

5. Wash out any traces of iodine in 85% alcohol.

6. Stain in orcein (solution No. 1) 2–24 hours but only long enough to color the elastic fibers a satisfactory red-brown. (If no elastic fibers are in the tissues or if one does not wish to stain elastic fibers this step may be omitted.)

7. Wash out excess of orcein in 2 changes of 85% alcohol and run slides down to water.

8. Stain in acid alizarin blue 5–10 minutes. (Solution No. 2).

9. Rinse in distilled water.

10. Decolorize and mordant in 5% aqueous phosphotungstic acid solution until collagen is destained, generally 10–30 minutes. (Fresh solution should be made up frequently.)

11. Rinse a few seconds in distilled water and stain 10 minutes in fast-green-orange-G (Solution No. 3).

12. Rinse thoroly in 50% alcohol and pass thru two changes of 95% alcohol, allowing a few minutes at least for each change. Parlodion-affixed sections may be left in alcohol until the stain comes out of the parlodion or nitrocellulose surrounding the sections.

13a. Pass parlodion or nitrocellulose sections from 95% alcohol thru two changes of terpineol, two changes of xylene, and mount in xylene-clarite.

13b. Pass paraffin sections from 95% alcohol thru two changes of absolute, two of xylene and mount in xylene-clarite (60% clarite lumps dissolved in xylene).

Results

Elastic fibers, red-brown; nuclei, basophilic granules, blue or purple; cytoplasm and muscle fibers, violet; collagen, reticulum or basement membranes, green; erythrocytes, myelin sheaths, acidophilic granules, plasmosomes, orange.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

OWENS, B. E. **An easily constructed stage micrometer.** *The Microscope*, 5, 95–6. 1942.

The author employs Dufray color film as a basis for a stage micrometer, because of the gelatin film, printed with a mosaic screen, on which its emulsion is coated. This screen consists of series of parallel red lines 1/500 in. apart superimposed at regular intervals on a series of alternate green and blue lines with reference to which the red lines run diagonally. The micrometer is constructed as follows: Cut a small piece of the film about ¼ in. square with its edges parallel to the red lines. Remove the silver grains forming the developed image which interfere with transparency; this is done by first soaking in water for 30 min. (to soften the gelatin), then for a few minutes in a mixture of 1 part of a 10% aqueous solution of $K_4Fe(CN)_6+3H_2O$ to 4 parts of a 20% solution of $Na_2S_2O_3+5H_2O$. When all silver grains have disappeared, wash the film well and allow it to dry. Mount in balsam, so oriented that the red lines (which are more clearly seen than the blue and green lines) are perpendicular to the length of the slide.

In use, the image of the mosaic slide is projected onto a piece of ground glass held in a wooden box which fits over the eyepiece of the microscope. This glass is divided by India ink lines ruled on the frosted side into ¼ in. squares.—*C. E. Allen.*

PHOTOMICROGRAPHY

LOVELAND, R. P. **Simplified photomicrography with a hand camera.** *Science*, 97, 24–6. 1943.

A method is described for using a box or folding hand camera for making good photomicrographs. Details are given for focusing, construction of apparatus (including figures), and exposure control.—*T. M. McMillion.*

MICROTECHNIC IN GENERAL

MACINTOSH, O. C. **Purification of paraffin.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 22, 99–100. 1942.

The curtailment of supplies of paraffin due to the war has made the purification of embedding paraffin advisable. The following procedure is recommended: Hold approximately 1000 cc. of the paraffin to be reclaimed overnight in an oven at 56°C. in an open flat container. Then heat over a bunsen burner to approximately 75°C. and keep at that temperature for 10 min. This eliminates the volatile impurities such as chloroform, acetone, formaldehyde, ethyl alcohol and a large part of the water. Next add 25 cc. of commercial Fuller's earth and stir well for 5 min. while maintaining the temperature at 75°C. Then filter the paraffin at 100°C. using a heated funnel of the hot water jacket type. This step removes pigments and products of tissue disintegration. Since xylene is difficult to eliminate, it is advisable to use chloroform instead in the preparation of sections if the paraffin is to be reconstituted. The resultant product has been found quite satisfactory for use in preparing ordinary tissue sections.—*Jean E. Conn.*

PACKER, D. M., and SCOTT, G. H. **A cryostat of new design for low temperature tissue dehydration.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **22**, 85–96. 1942.

A discussion is given of some of the principles of dehydration of frozen tissues as a preliminary to paraffin infiltration. An efficient apparatus for accomplishing frozen dehydration is described in some detail. There still remain a number of fundamental flaws in the technic among which are intracellular displacement and possible chemical alteration of some of the organic components occasioned by the freezing of the protoplasm.

The apparatus and methods described are a step toward perfecting the technic by removing all probability of the addition of water to the dried tissue.—*Jean E. Conn.*

RICHARDS, A. G., JR., ANDERSON, T. F., and HANCE, R. T. **A microtome sectioning technic for electron microscopy illustrated with sections of striated muscle.** *Proc. Soc. Exp. Biol. & Med.*, **51**, 148–52. 1942.

Sections varying in thickness from 0.1 μ to 0.2 μ were prepared by cutting specimens at a slight angle and drying them while frozen. Muscle tissue was fixed in alcohol, formalin, and acetic acid. Specimens were washed in water and transferred to "Carbo-Wax 4000" in distilled water. The wax was concentrated by evaporation of the water under a lamp; after which the specimen was transferred to a vessel of pure melted wax. Perpendicular cuts, with the knife edge set at 0.25 μ, were made with the surface of the specimen at a slight angle to the surface of the wax. Cut sections in a dry dish were passed thru moist air, picked up in distilled water, mounted on the wire disc used in the electron microscope and quickly frozen at −72°C. in petroleum ether and alcohol. They were dried under a vacuum while still frozen in 5–10 min.—*M. S. Marshall.*

DYES AND THEIR BIOLOGICAL USES

ANDERSON, G. W., FAITH, H. E., MARSON, H. W., WINNEK, P. S., and ROBLIN, R. O., JR. **Studies in chemotherapy. VI. Sulfanilamido heterocycles.** *J. Amer. Chem. Soc.*, **64**, 2902. 1942.

The preparation and properties of a number of new sulfanilamido heterocycles are described. Several of these compounds are closely related chemically to the well-known sulfanilamido derivatives of the same type. All of the sulfonamides showed some degree of bacteriostatic activity (*in vitro* tests against *Escherichia coli*) but very little effect against experimental streptoccal or pneumococcal infections or both in white mice. Two compounds (2-sulfanilamidoöxazole and 3-sulfanilamidopyridazine) were as active *in vitro* as sulfathiazole. In a discussion of the negative *in vivo* results, the author calls to the reader's attention the suggestion of Davis, that the activity of sulfonamides may be influenced by the binding effect of serum proteins and points out that his experimental observations only emphasize again the number of variables, both known and unknown, which may complicate any attempts to correlate *in vivo* activity with chemical structure.—*A. P. Bradshaw.*

BELL, P. H., and ROBLIN, R. O., JR. **Studies in Chemotherapy. VII. A theory of the relation of structure to activity of sulfanilamide type compounds.** *J. Amer. Chem. Soc.*, **64**, 2905. 1942.

Based on the experimental observation that the acid dissociation constants of N′- substituted sulfanilamide derivatives are related to their chemotherapeutic activity, a theory of the relation of structure to activity of these compounds is proposed. The acid and base constants of a large number of sulfanilamide type compounds have been determined. A plot of acid constants versus bacteriostatic activity, gives a smooth curve which passes thru a maximum as the acid strength increases. This correlation between acidic dissociation and activity is shown to be directly associated with the negative character of the SO_2 group. In brief, the theory may be stated as follows: the more negative the SO_2 group of a N′-substituted sulfanilamide derivative, the greater its bacteriostatic power. The inductive constants of the various N′- substituents have been evaluated. Based on this method, a quantitative treatment of the theory has been developed. The

calculated value of the acid constant for optimum activity agreed very well with the experimental results. The relative activity of the ionic and molecular forms of the sulfonamides has also been predicted by this treatment. Since acid constants are related to both the structure of the N′-substituent and the activity of the derivative, an indirect correlation between structure and chemotherapeutic activity is established. Knowing something about the relative electron attracting power of the N′-substituent it is possible for the first time to predict the bacteriostatic power of any new sulfanilamide derivative of this type. A discussion of the relation of structure to activity, and a description of the proposed theory and its implications are given.—*Author abstr.*

BURTON, H., McLEOD, J. W., MAYR-HARTING, A., and WALKER, N. **Development of colours from sulphonamides (*p*-nitrobenzenesulphonamide) under bacterial action and the bearing of such phenomena on the theory of the bacteriostatic and bactericidal activities of the sulphonamides.** *J. Path. & Bact.*, **54**, 407–19. 1942.

Chocolate agar containing 1:4000 of *p*-nitrobenzenesulphonamide will usually show a green zone around pneumococcal colonies and a purple zone around those of *Streptococcus hemolyticus*. Similar production of purple zones (occasionally masked by green colors) are elicited by many bacteria. The pigment is nondiffusible and appears only in blood agars. Irradiated solutions of sulphanilamide develop a similar color which appears to be associated with increased bactericidal effect.—*S. H. Hutner.*

FRANK, R. L., HOLLEY, W., and WIKHOLM, M. **3,2′-Nicotyrine. Insecticidal properties of certain azo derivatives.** *J. Amer. Chem. Soc.*, **64**, 2835. 1942.

Four azo dyes have been prepared by coupling 3,2′-nicotyrine with diazonium salts, namely, sodium 5′-(3,2′-nicotyrine)-azo-p-benzenesulfonate, 5′-(3,2′-nicotyrine)-azo-p-nitrobenzene, 5′-(3,2′-nicotyrine)-azo-p-benzoic acid and β-naphthaleneazo-5′-(3,2′-nicotyrine), which were tested for their activity as insect-proofing agents. Samples of wool dyed with the above compounds were found to be much less affected by the larvae of black carpet beetle than a sample of undyed wool. —*A. P. Bradshaw.*

GILBERT, H. W., and BLUM, H. F. **The mechanism of uptake of the dye, rose bengal, by the red cell.** *J. Cellular and Comp. Physiol.*, **19**, 257–70. 1942.

Rabbit red cells were washed three times in a solution of primary and secondary sodium phosphates of pH 6.7 and suspended in a like solution to which had been added the desired concentration of the dye. The concentrations of dye in the cells and in the suspending fluid were measured by centrifuging and determining the concentration of the dye in the supernatant liquid spectrophotometrically. The difference between the concentration so determined and the original concentration before the red cells were added gives the amount of dye taken up by the cells. The number of red cells was determined by a hemocytometer count.

The relationship of dye taken up per red cell to dye available per cell was found to be linear, the cell taking up approximately 72% of the available dye. The uptake of dye was reversible, with the return of the dye to the solution slower than the uptake. The total quantity of dye as well as the rate of uptake increased with an increase in temperature.

Three possible mechanisms of the uptake of the dye are discussed, the distribution between immiscible solvents, adsorption, and chemical reaction. From an analysis of the data obtained it is concluded that the adsorption mechanism is least probable, whereas the other two are more so. It is suggested that a probable explanation of the results obtained may be that both distribution between immiscible solvents and chemical combination participate in the uptake of the dye. —*L. Farber.*

NORTHEY, E. H., PIERCE, A. E., and KERTESZ, D. J. **Sulfanilamide derivatives. VIII. Sulfanilylamidines.** *J. Amer. Chem. Soc.*, **64**, 2763. 1942.

A series of sulfanilylamidines of the structure $4\text{-}(NH_2)C_6H_4SO_2N{=}C(R)N(R')R''$ were prepared where R was alkyl, aralkyl or heterocyclic and R′ and R″ were hydrogen, alkyl or heterocyclic. Disulfanilylamidines having the probable

structure, 4-$(NH_2)C_6H_4SO_2N=C(R)NHSO_2C_6H_4(NH_2)$-(4)′, were isolated as by-products. None of the compounds was more active than sulfanilamide in preliminary chemotherapeutic studies.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

MACINTOSH, O. C. **The preparation of transudates and exudates for paraffin embedding.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums,* 22, 97–8. 1942.

In the preparation of body fluids for paraffin embedding, difficulties have been encountered in removing the small amount of sediment obtained from the bottom of the centrifuge tube. To avoid this, small aluminum cups with a side measurement of 0.3 cm. have been devised to fit inside ordinary thick glass test tubes. The cup is placed in the tube with the concave side up, the fluid to be examined is poured in, and the tube is centrifuged for 20 min. After centrifugation the supernatant fluid is poured off, leaving the sediment in the cup which is then removed from the tube, covered with tissue paper, enclosed in a tissue button, and passed thru the various solutions. When ready for embedding, the paper cover is removed and the sediment is transferred with a small spatula to the embedding boat. The author claims that sections prepared in this way are much superior to fluid smears or fluid sediment prepared in the usual manner.—*Jean E. Conn.*

PONDER, ERIC, and PONDER, RUTH VAN OLINDA. **The cytology of the polymorphonuclear leucocyte in toxic conditions.** *J. Lab. & Clin. Med.,* 28, 316–22. 1942.

Stains are prepared as follows: Soak slides several hours in a strong solution of washing soda (e.g., Gold Dust). Rinse thoroly in hot running water, dip in alcohol and wipe dry. Make smears from fresh or heparinized blood, air-dry, fix in acetone-free methyl alcohol for 5 min. and air-dry. Stain with freshly diluted Giemsa stain (1:10 in distilled water) for exactly 30 min., rinse with running distilled water for 30 sec., drain, and dry in air. Examine with the oil immersion lens. Record, in terms of 0 to ++++, the findings for each of the following four cytological features of the polymorphonuclears: toxic granulation, ameboid contour, vacuolization of the cytoplasm, and pyknosis of the nucleii. Record the result as a grade of 0 to a maximum of 12; (12 would be ++++ for each cytological feature). This grade has a definite prognostic value.—*John T. Myers.*

MICROÖRGANISMS

DIENST, R. B. **The use of the fluoroscopic method for diagnosis of M. tuberculosis in sputum.** *Amer. J. Clin. Path., Tech. Suppl.,* 12, 61. 1942.

The fluoroscopic method was compared with the Ziehl-Neelsen technic for the detection of acid-fast bacteria in sputum. The fluoroscopic procedure used is as follows: Stain prepared smears 2–3 min. in a solution of 97 ml. of distilled water, 3 ml. of liquefied phenol and 0.1 g. of auramin 0 (National Aniline and Chemical Co.); wash in tap water; destain 3–5 min. in a fresh solution of 100 ml. 70% alcohol, 0.5 ml. conc. HCl and 0.5 g. NaCl; wash in tap water, dry and examine under the 4 mm. lens of a microscope equipped with fluorescence accessories (Spencer Lens Co.). Acid-fast organisms appear as small fluorescent rods against a nearly black background.

From an examination of 50 sputa from cases of tuberculosis and of samples of sputum from non-tuberculous patients by both methods, the author concludes that: 1) the Ziehl-Neelsen method is the more desirable one, since a picture of the cytology of the exudate is obtained at the same time; 2) both technics are equally efficient in detecting acid-fast bacilli in sputa from cases of tuberculosis; 3) sputa from non-tuberculous cases were diagnosed with more certainty when they were stained by the Ziehl-Neelsen method, since it was difficult to determine when the field was in focus in the fluoroscopic method.—*L. Farber.*

HUDDLESON, I. F. The presence of a capsule on Brucella cells. *Michigan State College Agr. Expr. Sta. Tech. Bull.* **177**, *Studies in Brucellosis*, 11–14. 1941.

By use of Keuffel and Esser (Keco) drawing ink No. 3000 it was possible to achieve negative staining of bacteria without "mucoid" artifacts originating from retraction of the ink from the bacterial surface. A satisfactory technic which includes staining of the cell bodies follows: Prepare a suspension of the *Brucella* cells in 0.85% NaCl or distilled water from a 24–48 hr. liver or tryptose agar culture. Place a small drop of the suspension near one end of a clean slide and draw across the slide by placing the end of another slide at one angle in front of the drop. Dry the bacterial film in air. Place a drop of ink back of the beginning of the previous smear and draw the ink over the smear with the end edge of another slide. Dry the inked smear in air. Treat the smear for 5 sec. with methyl alcohol acidified to pH 4.5 with glacial acetic acid. Air-dry. Place the slide, film side up, on a rack and stain for 2 min. with anilin oil crystal violet (Conn, H. J.: Biological Stains) previously diluted 1:1 with distilled water. Wash off excess stain with distilled water and dry smear in air.

The bacterial bodies appear deep purple inside the capsule surrounded by a light pink- or purple-staining substance. In old cultures the capsular area may be a clear empty space surrounded by a slight concentration of carbon particles. The procedure has been used successfully for *Pneumococcus*, streptococci, *Escherichia coli* and many other bacteria. The capsular material of many bacteria stains grayish green instead of pink.—*S. H. Hutner.*

LUZ, K., and MEDING, B. Über den fluorescenzmikroskopischen Nachweis von Tuberkelbazillen unter besonderer Berücksichtigung anderer alkoholsäurefester Bakterien. *Zentbl. Bakt., I Abt. Orig.*, **145**, 500–6. 1940.

The fluorescence technic by the acridine staining method, used since 1913 in botanical and zoological work, has been introduced in 1937 into the bacteriological laboratory technic, particularly in the examination for tuberculosis. The method saves time and facilitates the finding due to the better differentiating visibility of the acid-fast bacteria. An increased finding from 6.4% to 100% in comparison with the Ziehl-Neelsen method has been reported by different workers. The authors of this paper have investigated 1000 sputum smears and have found 154 positive with the regular Ziehl-Neelsen method. Enrichment with antiformin improved the result by 18 additional positive results. The fluorescence technic improved the positive results over the antiformin technic by 62 (36%). A differentiation between tubercle organisms and other acid-fast bacteria (smegma bacilli etc.) is not possible with this method, but can be obtained if the material is treated with chloroform for 5 min. or with 20% antiformin for 30 min. After this procedure only tubercle organisms are still stainable with acridine.—*Ivan Saphra.* (Courtesy Biological Abstracts.)

MEYRICK, LUCY D., and HARRIS, CHARLES V. A counterstain for use in Gram technique. *J. Path. & Bact.*, **54**, 517–8. 1942.

The suggested counterstain is very useful for identifying gonococci in smears because this organism apparently stains a much deeper red than do other gram negative bacteria. The procedure used is as follows: Stain in ammonium oxalate crystal violet and proceed as usual until counterstaining. Counterstain 1 min. with a mixture of 1% aqueous Ehrlich's neutral red, 15 vol., and carbol fuchsin (90 ml. 5% phenol + 9 ml. 10% alcoholic basic fuchsin), 1 vol. Rinse and dry as usual. The counterstain keeps well. The fuchsin component is an excellent bacterial and cytoplasmic stain but a poor nuclear stain; netural red is the reverse. The mixture combines the best features of each.—*S. H. Hutner.*

SNIESZKO, S. F. Detergents and staining bacteria. *Science*, **96**, 589. 1942.

A method is described which makes possible the preparation of satisfactory microscopic preparations of bacteria on mechanically cleaned slides. The method employs either a water solution of "Aerosol OT", 1:500, or a saline solution of "Aerosol MA", 1:500, one loopful of one of these being placed on the slide. Bacteria from liquid or solid media are added to the detergent and the suspension

spread with the loop. The preparation is then air-dried without heating, fixed with methyl alcohol or heat, and stained in the usual way. A comparable procedure for staining flagella is also described.—*T. M. McMillion.*

WEISS, EMIL. **A new method of staining tubercle bacilli.** *Amer. Rev. Tuberc.*, **46**, 199. 1942.

The author tested a number of different dyes for the primary staining of the organisms, various acids for the decolorization of the accompanying bacteria, and the use of mordants to enlarge the tubercle bacilli. From a consideration of the results obtained, the following staining technic is recommended: Prepare uniformly thin smears and, after drying, fix them over a flame; cover the slides with a mordant mixture (prepared by mixing 1 part of a solution of 100 g. tannic acid in 100 ml. 95% alcohol with 2 parts of a solution of 15 ml. glacial acetic acid diluted to 100 ml. with a 15% dilution of Merck's formaldehyde solution) and steam 5 min.; wash in running water; cover slides with a filtered solution of 2.5 g. safranin or pyronin in 100 ml. of 10% alcohol and steam 5 min.; wash in running water; decolorize with a solution of 15 ml. glacial acetic diluted to 100 ml. with acetone until colorless; wash well in running warm water; counterstain 1 min. with Loeffler's methylene blue (30 ml. of a saturated alcoholic solution of methylene blue, 5 g. in 100 ml. of 95% alcohol, diluted with 100 ml. of a 1:10,000 solution of KOH and filtered); wash with running water and allow to dry.—*L. Farber.*

YEGIAN, D., and BAISDEN, L. **Factors affecting the beading of the tubercle bacillus stained by the Ziehl-Neelsen technique.** *J. Bact.*, **44**, 667–72. 1942.

The production of beads in films of tubercle bacilli stained by the Ziehl-Neelsen technic is greatly influenced by the sample of basic fuchsin used in the staining process and by the concentration of the dye solution. With all the samples of fuchsin, addition of electrolyes greatly increased the number of beads. Washing the stained preparation with neutral alcohol, after it had been decolorized with acid alcohol, removed most if not all of the beads.

At a concentration of 0.3%, no beads were formed by the following dyes: pararosaniline hydrochloride (no lot number); basic fuchsin CE16 (rosaniline hydrochloride); basic fuchsin (rosaniline hydrochloride) No. 391205 of Coleman and Bell; and pararosaniline hydrochloride No. 9155 of National Aniline. A few beads formed with pararosaniline acetate No. 8772 and No. 6820 of National Aniline; more with pararosaniline acetate (no number) of Coleman and Bell; and many with basic fuchsin NF31, pararosaniline acetate of National Aniline. It is suggested that the beads may be precipitations of the dye on the surface of, or perhaps within, the bacteria.—*Virgene Kavanagh.*